The promiscuous evolutionary history of the family Bromoviridae

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INTRODUCTION

Recombination and segment reassortment are important contributors to the standing genetic variation of RNA viruses and are often involved in the genesis of new, emerging viruses. This study explored the role played by these two processes in the evolutionary radiation of the plant virus family Bromoviridae. The evolutionary history of this family has been explored previously using standard molecular phylogenetic methods, but incongruences have been found among the trees inferred from different gene sequences. This would not be surprising if RNA exchange was a common event, as it is well known that recombination and reassortment of genomes are poorly described by standard phylogenetic methods. In an attempt to reconcile these discrepancies, this study first explored the extent of segment reassortment and found that it was common at the origin of the bromoviruses and cucumoviruses and at least at the origin of alfalfa mosaic virus, American plum line pattern virus and citrus leaf rugose virus. Secondly, recombination analyses were performed on each of the three genomic RNAs and it was found that recombination was very common in members of the genera Bromovirus, Cucumovirus and Ilarvirus. Several cases of recombination involving species from different genera were also identified. Finally, a phylogenetic network was constructed reflecting these genetic exchanges. The network confirmed the taxonomic status of the different genera within the family, despite the phylogenetic noise introduced by genetic exchange.

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mechanisms by which RNA recombination may take place, the most common of which is homologous recombination (Lai, 1992). During this process, the donor replaces a highly homologous region in the acceptor, with the recombinant retaining exactly the same genomic organization of the parental RNA molecules. Therefore, the term homologous refers not only to the presence of sequence homology between both parental RNAs but also the necessity of homologous and comparable sites in both parental molecules for the crossovers to take place (Lai, 1992). aberrant homologous and non-homologous recombinations are less frequent in nature (Lai, 1992).

Amongst positive-strand plant RNA viruses, homologous recombination has been reported in vivo and in vitro during mixed infections (Bousalem et al., 2003; Cheng & Nagy, 2003; Moreno et al., 2004; Tan et al., 2004; Bonnet et al., 2005; Urbanowicz et al., 2005; Chare & Holmes, 2006; Weng et al., 2007), as well as in retroviruses such as human immunodeficiency virus type 1 (Worobey & Holmes, 1999; Prijic et al., 2004; Althaus & Bonhoeffer, 2005; Galetto & Negroni, 2005), although it is a rare or even non-existent phenomenon among negative-strand viruses (Chare et al., 2003). There are several
In addition to these recombination events, reassortment of genomic RNAs during mixed infection events of multipartite viruses is of extreme importance in the genesis of new strains. In certain cases, these new strains may show completely different virological properties, recent examples of which have been seen in H5N1 influenza A virus (Macken et al., 2006) and the begomovirus species complex responsible for the cassava mosaic disease (Lefevre et al., 2007). Segment reassortment has been observed among cucumoviruses (White et al., 1995), although the frequency of such heterologous segregations may be low (Fraile et al., 1997).

The Bromoviridae is one of the most important families of plant RNA viruses, with members distributed worldwide. It has a wide host range (more than 10 000 species) and is the cause of agronomically important diseases. The genome of members of the family Bromoviridae is composed of three segments of positive-sense, single-stranded RNA of approximately 8 kb. RNAs 1 and 2 are monocistronic and encode the proteins involved in virus replication (P1 and P2), whereas RNA 3 encodes the movement (MP) and coat (CP) proteins, the latter of which is translated from the subgenomic RNA 4. Some members of the family (especially cucumoviruses and some luteoviruses) encode a fifth protein (P2b) that is located in RNA 2 and is part of the C-terminal region of the P2 protein. This peptide is involved in silencing suppression, systemic movement and expression of symptoms (Ji & Ding, 2001; Shi et al., 2002).

In recent years, doubts have been cast on the biological significance of the currently accepted official taxonomy of the family Bromoviridae (Rampitsch & Eastwell, 1997; Sánchez-Navarro & Pallás, 1997; Scott et al., 1998; Shiel & Berger, 2000; Codoñer et al., 2005; Codoñer & Elena, 2006). To provide more clues for a better understanding of the taxonomy of this family, we undertook a systemic analysis of the potential roles of recombination and segment reassortment during mixed infections.

METHODS

RNA and protein sequences. Table 1 contains a list of virus species belonging to the family Bromoviridae that were used for this study. Sequences were obtained from the NCBI database (www.ncbi.nlm.nih.gov). Subgroups within the genus Ilarvirus were defined as in Codoñer & Elena (2006).

Protein sequence alignments were obtained using CLUSTAL_X v1.81 (Thompson et al., 1997). The alignments were adjusted manually to preserve previously described homologies and the domains defined in the Pfam HMM library (http://pfam.sanger.ac.uk), including the 30K domain characteristic of the MP (Melcher, 2000). RNA alignments were obtained by concatenating triplets according to the above amino acid alignment using DAMBE v4.2.7 (Xia & Xie, 2001). The 5' and 3' untranslated regions (UTRs) were aligned with CLUSTAL_X. Finally, all partial alignments for coding and non-coding sequences were appended to generate aligned RNA segments. Alignments are available upon request.

Phylogenetic reconstruction. A molecular evolutionary model was first fitted to each of the protein and RNA alignments using PROTEST v1.0.6 (Abascal et al., 2005) and MODELTEST (Posada & Crandall, 1998), respectively. For the MODELTEST analysis, PAUP v4.0b10 (Swofford, 1998) was used.

Phylogenetic trees for proteins were obtained using the PHYML program implemented in PROTEST that builds the best tree under the best molecular evolutionary model. Phylogenetic trees for RNA sequences inferred by the best model of nucleotide substitutions were obtained using the CODEML program from PAML v4.0 (Yang, 2007). We accepted the best tree as the one explaining the evolution of each RNA segment with the lowest log-likelihood value. CODEML was also used to obtain the log-likelihood value for the different trees.

RNA segment reassortment analyses. To look for RNA segment reassortment during mixed infections, a phylogenetic congruence analysis was performed. If segments have co-speciated, i.e. always segregated together, then the tree topologies obtained from different segments must be congruent. In contrast, if reassortment events have taken place during the evolution of the family, the tree topologies will show incongruence among segments. Non-random associations between all three possible pairs of RNAs were determined by reconciling the corresponding phylogenies using a heuristic search in TREemap v1.0a (Page, 1994). This approach minimizes the number of independent segregation events among virus segments. The statistical significance of the phylogenetic associations was assessed by performing 10 000 randomizations of both trees using the proportional-to-distinguishable method (Page, 1994). Four different processes affect the relationship between segments (Page, 1994): (i) a co-speciation event takes place when parallel cladogenesis occurs for all segments, i.e. the split of an ancestral species into two new ones maintains the linkage between all three segments; (ii) a duplication event occurs when cladogenesis does not affect all segments at the same time, i.e. after the split of an ancestral species into two new ones, one of the segments speciates but the other remains common among the two new species; (iii) a switch event takes place when a segment from one virus is acquired by a different one; a priori, this should be neglected as no case of gaining an extra segment has been reported; (iv) a sorting event has happened when segments differentially sort during a co-infection process; this type of event is the key support for the reassortment hypothesis.

Recombination analyses. In addition to reassortment events, recombination within a segment can also seriously influence the branching order and branch lengths of phylogenetic trees (Posada & Crandall, 2002). The presence of recombination events within each RNA segment was explored using RDP v3.22/ (Martin et al., 2005b). RDP incorporates several published recombination detection methods into a single suite of tools. Once all potential recombination events are identified, RDP sorts the results and determines the number of unique recombination events identifiable, determining the daughter and parental sequences and the break points. The programs used in this study were: RDP (Martin & Rybicki, 2000), GENECONV (Padidam et al., 1999), BOOTSCAN (Martin et al., 2005a), MAXCHI (Smith, 1992), CHIMAERA (Posada & Crandall, 2001), SISCAN (Gibbs et al. 2000), 3SEQ (Boni et al., 2007) and LARD (Holmes et al., 1999). In all cases, default parameters were used. Only events predicted by half of the methods were considered as significant; this ensured that our approach remained conservative.

The likelihood of predicted recombination events is discussed with regard to the possibility of co-infesting the same host plant by the parental viruses. A list of susceptible hosts for each virus can be obtained from the Plant Virus Online database (http://image.fs.uida-ho.edu/vide/refsh.htm).

Phylogenetic networks. Because reassortment and recombination produce networks of sequences rather than strictly bifurcating evolutionary trees, as a final step in our analysis a phylogenetic
network (Huson & Kloepper, 2005) was constructed from the concatenated alignment of the three genomic RNA segments. This network, obtained by the split decomposition method implemented in SPLITSTREE v4.8 (Huson & Bryant, 2006), depicts parallel edges between sequences when there are conflicting phylogenetic signals.

RESULTS AND DISCUSSION

Fig. 1 shows the phylogenetic trees inferred for the three segments by concatenating the corresponding coding and non-coding regions. A single phylogenetic tree per RNA segment was inferred using CODEML and fitting of heterogeneous substitutions models was carried out as described in Codóner et al. (2005). The three topologies were not fully congruent. Within the genus Ilarvirus, the association of AMV with PDV (see Table 1 for virus abbreviations) depended on which RNA was analysed: according to RNAs 1 and 3, these two viruses share a common phylogenetic origin whereas, according to RNA 2, PDV is a closer relative of PNRSV and ApMV. The location of CiLRV inferred from RNA 3 was also incongruent with that inferred from the other two RNAs. The location of APLPV was also incongruent: it appeared within the genus Ilarvirus according to RNAs 1 and 3 but clustered outside the genus according to RNA 2. Similarly, the order of clustering within the genus Cucumovirus depended entirely on which RNA was analysed. Finally, the clustering obtained for the three bromoviruses was congruent for RNAs 2 and 3, placing CCMV and BBMV as closer relatives, but clustering with RNA 1 was incongruent.

Segment reassortment during evolution of the Bromoviridae

A possible explanation for the phylogenetic discrepancies found is the existence of segment reassortment events during the phylogenetic radiation of the family. Such independent segregation events may take place during co-infection of a common host by two or more viruses. To test for the existence of such non-co-speciation events, the methods implemented in TREEMAP were applied on a pairwise basis. Fig. 2 shows the reconciliated trees for each pair of genomic segments together with a scatter plot illustrating the positive correlation between the genetic distances estimated for the branches where co-speciation events took place. In the case of RNAs 1 and 2, the reconciled tree that required the smallest number of independent segregation events was still compatible with five duplication and 17 reassortment events (resampling test: P = 0.0001). In those 12 cases where co-speciation took place, the rates of genetic evolution of both segments were positively and significantly correlated (Fig. 2a: r^2 = 0.9129, P < 0.0001). This indicates that the evolution of both segments was not independent and that accelerations and decelerations in one RNA segment are associated with equivalent events in the other. Reassortment events concentrated on two branches of the reconciliated tree. Reassortment is likely to have happened firstly during the genesis of APLPV, AMV and PDV, and secondly in the complex origin of the bromoviruses and cucumoviruses (Fig. 2a).

Table 1. Members of the family Bromoviridae included in the analysis

Assigntion to different subgroups within the genus Ilarvirus follows Codóner & Elena (2006).

<table>
<thead>
<tr>
<th>Genus</th>
<th>Virus</th>
<th>GenBank accession no.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RNA 1 (P1) RNA 2 (P2) RNA 3 (MP and CP)</td>
<td></td>
</tr>
<tr>
<td>Bromovirus</td>
<td>Broad bean mottle virus (BBMV)</td>
<td>NC_004008 NC_004007 M60291</td>
</tr>
<tr>
<td></td>
<td>Brome mosaic virus (BMV)</td>
<td>NC_002026 NC_002027 X58458</td>
</tr>
<tr>
<td></td>
<td>Cowpea chlorotic mottle virus (CCMV)</td>
<td>NC_003543 NC_003541 M28818</td>
</tr>
<tr>
<td>Cucumovirus</td>
<td>Cucumber mosaic virus (CMV)</td>
<td>NC_002034 NC_002035 D00385</td>
</tr>
<tr>
<td></td>
<td>Peanut stunt virus (PSV)</td>
<td>NC_002038 NC_002039 D00668</td>
</tr>
<tr>
<td></td>
<td>Tomato aspermy virus (TAV)</td>
<td>NC_003837 NC_003838 L15335</td>
</tr>
<tr>
<td>Ilarvirus</td>
<td>Tobacco streak virus (TSV)</td>
<td>NC_003844 NC_003842 X00435</td>
</tr>
<tr>
<td>(subgroup 1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Citrus leaf rugose virus (CiLRV)</td>
<td>NC_003548 NC_003547 U17390</td>
</tr>
<tr>
<td></td>
<td>Elm mottle virus (EMoV)</td>
<td>NC_003569 NC_003568 U85399</td>
</tr>
<tr>
<td></td>
<td>Spinach latent virus (SpLV)</td>
<td>NC_003808 NC_003809 U93194</td>
</tr>
<tr>
<td></td>
<td>Tulare apple mosaic virus (TAMV)</td>
<td>NC_003833 NC_003834 AF226162</td>
</tr>
<tr>
<td>(subgroup 2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Apple mosaic virus (ApMV)</td>
<td>NC_003464 NC_003465 U15608</td>
</tr>
<tr>
<td></td>
<td>Prunus necrotic ringspot virus (PNRSV)</td>
<td>NC_004362 NC_004363 Y07568</td>
</tr>
<tr>
<td></td>
<td>Prune dwarf virus (PDV)</td>
<td>U57648 AF277662 L28145</td>
</tr>
<tr>
<td></td>
<td>American plum line pattern virus (APLPV)</td>
<td>NC_003451 NC_003452 AF235166</td>
</tr>
<tr>
<td></td>
<td>Alfalfa mosaic virus (AMV)</td>
<td>NC_001495 NC_002042 M59241</td>
</tr>
<tr>
<td>Oleavirus</td>
<td>Olive latent virus 2 (OLY)</td>
<td>NC_003673 NC_003674 X76993</td>
</tr>
<tr>
<td>Unassigned</td>
<td>Pelargonium zonate spot virus (PZSV)</td>
<td>NC_003649 NC_003650 AJ272329</td>
</tr>
<tr>
<td>Tobamovirus</td>
<td>Tobacco mosaic virus (TMV)</td>
<td>NC_001367 NC_001367 NC_001367</td>
</tr>
</tbody>
</table>
Fig. 2(b) shows the best reconciled tree for RNAs 1 and 3. In this case, the tree was compatible with six duplication and 20 reassortment events (resampling test: $P<0.0001$). As with the previous comparison, reassortment events mostly took place during the split of the bromoviruses and cucumoviruses from their last common ancestor; and during the diversification of APLPV, AMV and PDV. In this case, two extra reassortment events explain the incongruence observed for CiLRV RNA 3. Interestingly, it is necessary to assume an ancestral duplication event to explain the evolutionary origin of PZSV RNA 3. This ancestral segment was not shared by any other member of the family. In the 11 cases where co-speciation between RNAs 1 and 3 occurred, the rates of evolution were positively correlated (Fig. 2b: $r^2=0.7692$, $P<0.0001$). Indeed, supporting this hypothetical origin for PZSV RNA 3 and diminishing the likelihood of an origin outside the family. Interestingly, the RNA 3 sequence showed significant homology with two sequences from the other two PZSV segments: (i) nt 2401–2654 showed 94% identity with nt 3106–3383 of RNA 2 (BLASTN score: $E=2 \times 10^{-95}$); and (ii) nt 2384–2659 showed 89% identity with nt 2181–2435 of RNA 1 ($E=10^{-105}$).

Fig. 2(c) shows the best reconciled tree for RNAs 2 and 3. Twelve co-speciation events were predicted in this case and the positive correlation between the rates of genetic divergence in the two RNAs suggested concerted evolution in these particular branches (Fig. 2c: $r^2=0.6439$, $P<0.0001$). Nonetheless, five duplication and 17 reassortment events were required to reconcile both phylogenetic trees (resampling test: $P<0.0001$).

Pooling together the information from these three analyses, the following picture emerges, showing the importance of reassortment events on the genesis of new viral species. (i)
Assortment and recombination in the Bromoviridae

(a) RNA 1 vs RNA 2

(b) RNA 1 vs RNA 3

(c) RNA 2 vs RNA 3
The origin of bromoviruses and cucumoviruses is quite promiscuous, with many such reassortment events. For example, BMV RNA 1 was generated by duplication of an ancestral segment and did not follow the cladogenetic pattern of the other two segments. To explain the evolution of the cucumoviruses, an ancestral duplication and three reassortment events are required and, consequently, different RNAs support a different clustering. (ii) The origin of PZSV RNA 3 is a very old duplication event that took place in the last common ancestor of the family. (iii) CiLRV RNA 3 was recruited from the last common ancestor of SpLV and EMoV and did not co-speciate with RNAs 1 and 2 of TAMV. (iv) AMV RNA 2 was recruited from the common ancestor of PNRSV and ApMV and, as such, reflects a different evolutionary history from their corresponding RNAs 1 and 3, which had indeed co-speciated. (v) The origin of APLPV RNA 1 is consistent with at least one reassortment event in which the segment was obtained from a donor related to the last common ancestor of AMV and PDV.

Recombination events during evolution of the Bromoviridae

In addition to the above-discussed independent segregation events during the diversification of the family, we looked for fingerprints of recombination events within segments as an additional source of phylogenetic noise. Table 2 shows the results of the analyses performed with the RDP program. Only recombination events significantly supported by at least three different methods have been included in the table.

One recombination event was identified in RNA 2 (Table 2). The fragment nt 3130–3756 (3' end of the P2 coding gene) of AMV comes from a recombination event between PNRSV and PDV, PDV being the parental donor for this small segment. Both viruses infect Prunus spp., providing ample opportunity for co-infection, which may have created the arena for recombination.

Six recombination events were detected in RNA 3 (Table 2). The first three were involved in the origin of CiLRV RNA 3. The 5' and 3' ends of CiLRV were donated by TAMV (from nt 1 to 501, including the 5' UTR and the 5'-proximal part of MP, and from nt 3040 to the end of the segment, including the 3' end of CP and the 3' UTR). The region encompassing nt 1763–2026 (CP gene) of CiLRV RNA 3 was the result of a potential recombination event in which EMoV acted as the minor parental donor. In both cases, SpLV acted as the major parental donor. EMoV and SpLV share susceptible hosts in seven plant families (including Cucurbitaceae, Leguminosae and Solanaceae), whereas TAMV and SpLV also share susceptible hosts in the Leguminosae and Solanaceae. Therefore, mixed infections between these three viruses may have taken place. The fourth recombination event also involved CiLRV, SpLV and TAMV, although in this case CiLRV acted as the minor parental donor for the fragment nt 1982–2889 in the TAMV CP gene. SpLV and CiLRV only share susceptible hosts in the family Leguminosae. The fifth potential recombination event involved the 3' part of MP and the 5' part of the CP genes (nt 1198–2050) of the three bromoviruses. In this case, BMV is the recombinant virus, CCMV the major parental donor and BBMV the minor donor.

Table 2. Inferred most likely recombination events.

Only events supported by at least three different methods are reported. The support probability for each method is shown. Nucleotide numbering corresponds to the aligned sequences.

<table>
<thead>
<tr>
<th>Recombinant virus</th>
<th>Putative parental (major×minor)</th>
<th>Fragment (nt)</th>
<th>Inference method (P value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNA 2 AMV</td>
<td>PNRSV × PDV</td>
<td>3130–3756</td>
<td>RDP (2.07 × 10⁻⁵), BOOTSCAN (3.69 × 10⁻²), MAXCHI (4.55 × 10⁻⁵), CHIMAERA (6.98 × 10⁻⁴), SISCAN (1.63 × 10⁻⁷)</td>
</tr>
<tr>
<td>RNA 3 CiLRV</td>
<td>SpLV × TAMV</td>
<td>1–501, 3040–end</td>
<td>RDP (1.73 × 10⁻⁴), BOOTSCAN (2.35 × 10⁻³), MAXCHI (2.91 × 10⁻⁴), CHIMAERA (3.37 × 10⁻⁴), SISCAN (2.79 × 10⁻¹²)</td>
</tr>
<tr>
<td></td>
<td>SpLV × EMoV</td>
<td>1763–2026</td>
<td>RDP (9.27 × 10⁻³), BOOTSCAN (1.15 × 10⁻²), MAXCHI (4.18 × 10⁻²), CHIMAERA (1.93 × 10⁻²), SISCAN (2.46 × 10⁻²)</td>
</tr>
<tr>
<td>TAMV</td>
<td>SpLV × CiLRV</td>
<td>1982–2889</td>
<td>RDP (1.31 × 10⁻⁵), GENECONV (8.01 × 10⁻⁵), MAXCHI (6.60 × 10⁻⁷), CHIMAERA (2.66 × 10⁻⁴), SISCAN (4.86 × 10⁻⁵), 3SEQ (9.71 × 10⁻⁵)</td>
</tr>
<tr>
<td>BMV</td>
<td>CCMV × BBMV</td>
<td>1198–2050</td>
<td>RDP (4.13 × 10⁻⁴), MAXCHI (2.09 × 10⁻⁴), CHIMAERA (4.98 × 10⁻⁴), SISCAN (4.02 × 10⁻⁴)</td>
</tr>
<tr>
<td>PSV</td>
<td>TAV × PNRSV</td>
<td>239–505</td>
<td>RDP (1.32 × 10⁻⁴), BOOTSCAN (7.15 × 10⁻⁷), MAXCHI (1.06 × 10⁻⁴), CHIMAERA (6.83 × 10⁻³), SISCAN (1.27 × 10⁻⁸), 3SEQ (4.14 × 10⁻³)</td>
</tr>
</tbody>
</table>
one. The opportunity for mixed infection between both parental viruses exists, as both share susceptible hosts in four plant families (Amaranthaceae, Chenopodiaceae, Leguminosae and Solanaceae). The remaining putative event involved members of different genera: the region of nt 239–505 (part of the 5' UTR and the 5' most proximal part of MP) of PSV was predicted to be donated by PNRSV, whereas the rest of its RNA 3 resembled that of TAV. Plant species from four families (Compositae, Cucurbitaceae, Leguminosae and Solanaceae) are susceptible to TAV and PNRSV.

**A phylogenetic network to describe the evolution of the Bromoviridae**

Fig. 3 shows the unrooted phylogenetic network obtained for the concatenated alignment of the three RNA genomic segments. This network depicts a complex evolutionary scenario in which recombination and reassortment events play a fundamental role during the diversification of the family. Remarkably, the ilarviruses constitute a homogeneous group that share only two of such exchange events with the other genera, although many potential instances of genetic exchange among species have taken place within the genus, as indicated by a highly connected network among the different members of the genus. The genera Bromovirus and Cucumovirus are clearly separated, although the amount of gene flow among their members is also high, as illustrated by the dense connectivity of the network in their neighbourhood.

Several taxonomic implications can be drawn from Fig. 3. Firstly, as previously reported by several authors, based upon the analyses of RNAs 1 and 2 (Rampitsch & Eastwell, 1997; Scott *et al.*, 1998; Shiel & Berger, 2000) and RNA 3 (Sánchez-Navarro & Pallás, 1997; Codoñer *et al.*, 2005), and recently from the whole proteome (Codoñer & Elena, 2006), AMV does not deserve the status of an independent genus but must be considered as one more member of the genus ilarvirus. Secondly, the location of PZSV in the network, closer to the highly connected cluster formed by members of the genera Bromovirus and Cucumovirus, supports its inclusion in the family. It has recently been suggested that a new genus should be created, Anulavirus, in which PZSV would be the only current member (Gallitelli *et al.*, 2005). The precise location in the network, outside any other genera, supports this hypothesis. Thirdly, using the same argument, the validity of the genus Oleavirus is confirmed.
In this study, we were not concerned primarily with the role of selection during the genetic diversification of the different members of the family Bromoviridae. It is likely that selection has played an important role in shaping the genomes of the different viruses collectively known today as members of the family Bromoviridae. As is the case for point mutations, segment reassortment and homologous recombination create new genetic variability on which selection may operate. For example, positive selection may have been driving adaptation to new hosts, whereas negative selection may have been acting on removing genetic variability that produces deleterious effects in the host where it arose. In this study, we have shown that both segment reassortment and recombination have been frequent phenomena in the genesis of new viral species. Obviously, only those new combinations able to replicate in a given host would survive purifying selection and contribute to the next generation. In this respect, the reassortment and recombination events described here were viable ones as they all passed the filter imposed by purifying selection. Many other combinations did not succeed and hence are not detectable today. Immediately after this first selective filter, directional selection starts playing a role in which the interaction between the different parts of the newly created mosaic genomes is finely tuned. In this sense, co-evolution at the molecular level has been detected not only between amino acids belonging to the same protein but also among domains of the MP and CP proteins of PNRV (Codoñer et al., 2006), proving that selection does not always act on individual amino acids but usually does so on the interactions between multiple amino acids or even entire domains.

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